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Effects of Decreased Salinity on Expulsion of Zooxanthellae in the Symbiotic Sea Anemone *Anthopleura elegantissima*[1]

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ABSTRACT: Many natural conditions cause expulsion of zooxanthellae from corals and sea anemones. Recent studies have focused on causes and mechanisms of this release. We examined an incidence of bleaching in a field population of the sea anemone *Anthopleura elegantissima* (Brandt). Our data suggest that expulsion of zooxanthellae was caused by reduced salinity from freshwater runoff after heavy rainfall. In the laboratory, *A. elegantissima* expelled zooxanthellae in quantities directly correlated with strength and duration of exposure to hyposalinity. The mechanism of release appears to be rupture of the host cell, followed by accumulation of clumps of zooxanthellae that are then expelled from the coelenteron. *A. elegantissima* has little or no ability to osmoregulate the water in its coelenteron, and internal salinity drops rapidly with external salinity reduction.

MANY CNIDARIANS RELY on symbiotic zooxanthellae. Much of the carbon photosynthetically fixed by zooxanthellae is translocated to the host, satisfying a large part of the cnidarian's nutritional needs (Fitt and Pardy 1981, Falkowski et al. 1984). However, cnidarians may expell the zooxanthellae in the presence of various environmental simuli, including changes in water temperature, decreased salinity, and high levels of sunlight (Steen and Muscatine 1987, Muscatine et al. 1991).

The sea anemone *Anthopleura elegantissima* (Brandt), a symbiotic cnidarian, withstands many harsh conditions in the intertidal environment, including sand coverage, prolonged exposure to air, or weeks of starvation (Fitt et al. 1982, Taylor and Littler 1982). However, *A. elegantissima* in the laboratory expells its zooxanthellae during prolonged darkness, high temperatures (Buchsbaum 1968), and long-term exposure to bright sunlight (Dykens and Shick 1984). Other sea anemones show similar responses when exposed to high salinity (Reimer 1971), decreased temperatures (Steen and Muscatine 1987), a cold shock (Muscatine et al. 1991), or UV radiation (Lesser and Shick 1989).

Symbiotic *A. elegantissima*, found along the North American Pacific coast from Alaska to southern California, ranges in color from bright green, with zoochlorellae as symbionts, to greenish brown, containing zooxanthellae. Aposymbiotic, algae-free anemones appear white. Aposymbiotic *A. elegantissima* occurs in nature in fully shaded environments such as in caves and under wharves, deep within mussel beds, and occasionally in the high intertidal if continually exposed to direct sunlight (Buchsbaum 1968). Color is indicative of the anemone's symbiotic state, but it is not a perfect guide to the presence or absence of zooxanthellae, because aposymbiotic anemones may appear green or brown from endogenous animal pigments (Buchsbaum 1968).

Expulsion of zooxanthellae as a response to decreased salinity has not been observed

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previously in *A. elegantissima*. However, after naturally occurring decreases in salinity, reef corals in Jamaica and Easter Island expelled zooxanthellae (Goreau 1964, Egana and DiSalvo 1982). Reef corals exposed to decreased salinity in the laboratory were less tolerant of increased temperatures and bright light (Coles and Jokiel 1978).

In March of 1992 we observed that many *A. elegantissima* in the intertidal area of Leo Carrillo State Beach in Los Angeles County, California, were white, suggesting loss of zooxanthellae. These anemones were located near an arroyo that inundated this area of the intertidal zone with fresh water repeatedly during the unusually heavy winter rains of 1991–1992. Nearby rocky intertidal areas that did not experience inundations contained few white and numerous brown *A. elegantissima*.

The heavy winter rains of 1991–1992 occurred primarily from December to March, with a total of 56 cm of rain falling during that 4-month period. The runoff after each rain continued for many days. The days in February immediately following the heaviest rains were days with minus low tides. In contrast, the previous 7 yr had been a time of drought. Only 28 cm of rain fell during December to March in the 1990–1991 winter, and that rainfall was dispersed, not concentrated in several heavy rains as in the 1991–1992 season.

We hypothesized that decreased salinity during flooding with freshwater runoff caused the *A. elegantissima* at Leo Carrillo State Beach to expel zooxanthellae. We planned a series of field and laboratory measurements to test this hypothesis, including comparison of the color and numbers of anemones at this site with those at a control site, comparison of the numbers of zooxanthellae contained in the tissues of white and brown anemones, laboratory observation of the effect of reduced salinity on *A. elegantissima* and its zooxanthellae, and microscopic examination of recently expelled zooxanthellae. This investigation provides new information on natural occurrences of aposymbiotic anemones and the effects of decreased salinity on *A. elegantissima*.

MATERIALS AND METHODS

Field Sites

Leo Carrillo State Beach (LCSB) and Latigo Bay (LB) are both located in Los Angeles County, California, at 34° 02′ N, 118° 54′ W, and 34° 02′ N, 118° 46′ W, respectively. Both sites contain boulder fields that create a series of shallow tide pools during low tides. Numerous *A. elegantissima* occur in the pools and on the boulders. Both beaches are waveswept, located directly east of rocky points. A broad freshwater drainage, the Arroyo Sequit, empties onto the beach at LCSB during and after rains. LB, 26 km east of LCSB, has a much smaller point source of rainwater runoff, a single drainpipe that opens onto a sandy portion of the beach distant from the rocky point.

Field Measurements

Field data were collected during minus tides in June and July 1992. Number and color of *A. elegantissima* and salinity of the water were assessed in transects at both sites that were randomized in distribution and 1 m^2 in size. At LCSB a line transect parallel to the shore at a tidal height of -0.15 m was sampled at 3-m intervals for number and color of *A. elegantissima* per square meter in the boulder field within (ca. 60 m) and on either side (ca. 65 m total) of the area affected by freshwater inflow.

Numbers of brown and white anemones were compared between the control and runoff sites by chi-square test, and numbers of white anemones per transect were compared by correlation analysis to salinity levels. Number of anemones per transect within and out of the runoff area were compared by student's t test. A probability of <0.05 was considered a significant difference.

Collection and Maintenance of Experimental Organisms

Anthopleura elegantissima was collected from LB and LCSB during minus tides and individually labeled in finger bowls immersed in aerated seawater at ambient ocean conditions, salinity 32‰, 16°C, in a 14 : 10 (L : D) h photoperiod (30 μE m^{-2} sec^{-1}). They were fed *Artemia* nauplii weekly, except during experimental treatments. After the studies, anemones were returned to the field sites.

Estimation of Numbers of Zooxanthellae

Brown *A. elegantissima* from LB were used as controls and in hyposalinity experimental treatments. In addition, 10 *A. elegantissima*, five pale brown and five white, were collected from LCSB for estimation of numbers of zooxanthellae. All anemones were between 5 and 10 cm in column diameter. To count zooxanthellae, we used a method modified from Steen and Muscatine (1987). We removed and weighed several tentacles from an anemone to the nearest 0.001 g, diluted the tissue 1 : 10 (wt/vol) with sterile seawater, and homogenized it thoroughly. The solution was centrifuged for 5 min at 4000 rpm to separate algae from animal tissue, and then the pellet was resuspended in 1 : 10 (wt/vol) seawater by vigorous stirring. The zooxanthellae were counted on a hemacytometer slide. This technique allows calculation of the number of zooxanthellae per gram of excised tissue. Using tentacles instead of whole animals permitted serial measurements on the same individuals. The density of symbiotic algae varies with the location in the host's body, but is highest in the tentacles (G. Muller-Parker, pers. comm.). Although this technique is invasive, it is not lethal.

Numbers of zooxanthellae per gram wet weight were compared for animals from the control and runoff sites by student's t test.

Exposure to Decreased Salinity

Initial counts of zooxanthellae of symbiotic *A. elegantissima* from LB were made while the anemones remained in aquaria containing aerated seawater, 32‰, at 16°C. Deionized water at 16°C was added, over a period of 3 hr, to create a freshwater (FW): seawater (SW) ratio that was either 25 : 75% (24‰), 50 : 50% (16‰), or 75 : 25% (8‰) FW : SW, depending on the treatment. All anemones were maintained under lights as above. Control organisms were assessed for numbers of zooxanthellae and were maintained in aerated, 16°C seawater for the same period of time as experimental treatments. In the 24‰ treatment, anemones remained immersed in the hyposaline water for 21 days; zooxanthellae were counted on days 7, 14, and 21. Before each intermediate count and after the treatments, anemones were returned to full-strength seawater for several hours to facilitate the removal of tentacles for counting zooxanthellae. The oral disk, which often remained tightly closed during the entire hyposaline treatment period, opened within 3 to 24 hr after the return to seawater.

Counts were graphed over time for each salinity, and time to loss of 50% of the initial zooxanthellae was computed from these data.

Coelenteron Salinity

Three experiments were conducted. In the first, *A. elegantissima* ($n = 4$) was removed from seawater and placed directly in water with 24‰ salinity. Water samples of 0.1 ml were taken every 15 sec by inserting a 26-gauge needle carefully into the mouth and drawing out 0.1 ml with a 1-ml syringe. The procedure was repeated until the salinity within the coelenteron equaled external salinity. A second experiment ($n = 3$), in which external salinity was decreased gradually, resulted in a salinity of 7‰ after 35 min. Samples of coelenteron fluid were taken during this salinity reduction at 2-min intervals. To ascertain whether this removal of internal water accelerated the rate at which the coelenteron salinity decreased, a third experiment was conducted on anemones ($n = 3$) in 16‰ water. A single 0.1-ml sample was taken after 15 sec, and then the anemones were returned to seawater. The anemones remained in full-strength seawater until the internal salinity returned to 32‰. Then they were reimmersed in the 16‰ solution, and a second coelenteron sample was taken after 30 sec. The animal was returned to full-strength seawater. The procedure was repeated, and the interval lengthened until coelenteron salinity reached external salinity.

Host Cell Condition: Epifluorescence Microscopy

Anthopleura elegantissima ($n = 3$) was placed in 24‰ salinity water for 3 days, during which time the oral disk remained tightly closed. When returned to 100% seawater, the anemones expanded the oral disk and released large clumps of expelled zooxanthellae. These clumps were collected by pipette and examined to compare with excised tentacles taken from healthy *A. elegantissima* as controls. We placed samples onto slides (ProbeOn Plus, Fisher Scientific Products) that were charged and precleaned and stained them with fluorescein diacetate dye (stock solution 15 mg/ml in acetone; working solution 0.04 ml in 9.96 ml 0.1 M sodium phosphate, 3% sodium chloride, 0.004% calcium chloride, pH 7.4 [Gates et al. 1992]). The slides were rinsed twice with phosphate buffer and then viewed for epifluorescence with a microscope (Olympus IMT-2) with epifluorescence illuminator equipped with a BP490 exciter filter, dichroic mirror DM500, and barrier filter AFC0515.

RESULTS

Field Measurements

There were significantly fewer *A. elegantissima* per square meter transect at LCSB, 7.45 ± 4.73 m^{-2}, than at LB, 27.3 ± 13.0 m^{-2} ($P < 0.001$; t test). All ($n = 820$) of the *A. elegantissima* in random transects at LB were brown, indicating the presence of symbionts, but only 45% of the *A. elegantissima*

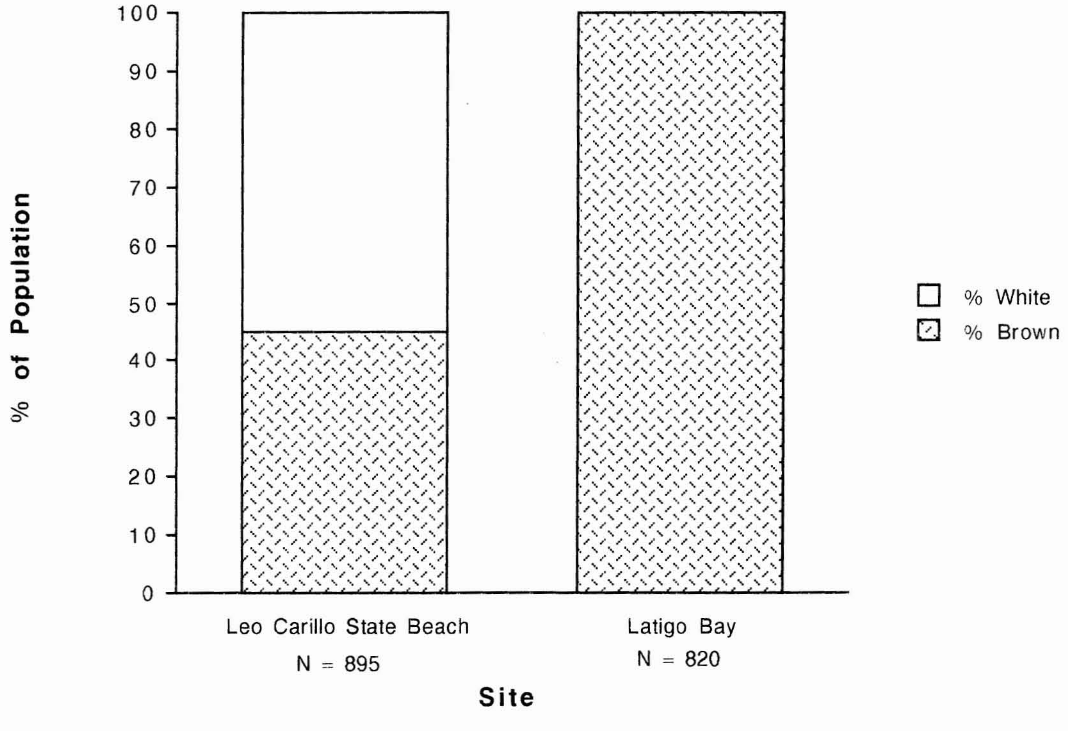

FIGURE 1. Distribution of white and brown *A. elegantissima* at the affected site, Leo Carillo State Beach, and a control site, Latigo Bay. The distribution of bleached and brown anemones is significantly different ($P < 0.001$) at the two sites (chi-square test).

($n = 895$) in random transects within the freshwater influx area at LCSB appeared brown (Figure 1). While walking through the LB site, we observed a total of five pale *A. elegantissima*, but none occurred among the 820 anemones counted in the transects, a significant difference in distribution by chi-square test ($\chi^2 = 992$, df = 1, $P \ll 0.001$). Salinity in the intertidal zone of LCSB during the summer low tides was not correlated with increased percentage of white *A. elegantissima* in random transects ($R^2 = 0.09$).

Density of brown *A. elegantissima* in transects within the area of freshwater influx at LCSB was not significantly different ($P > 0.05$) from densities of brown anemones on either side of this area, although those within the runoff area appeared paler brown, whereas the numbers of white anemones within the freshwater influx area were significantly higher ($P < 0.01$) than outside this area (Figure 2).

Comparison of Color and Number of Zooxanthellae

The white anemones from LCSB, although they appeared bleached, still contained some zooxanthellae, $4.29 \pm 3.40 \times 10^6$ zooxanthellae g^{-1} wet weight of tentacle. The pale brown anemones from LCSB had $4.93 \pm 3.07 \times 10^6$ zooxanthellae g^{-1} wet weight of tentacle (Figure 3). These two samples, white and pale brown, were not significantly different, so were pooled for a LCSB freshwater-influenced population mean of $4.61 \pm 3.43 \times 10^6$ zooxanthellae g^{-1}. Thus, color of anemones in the field at LCSB was not a reliable indicator of algal density. Anemones from LB were deeper

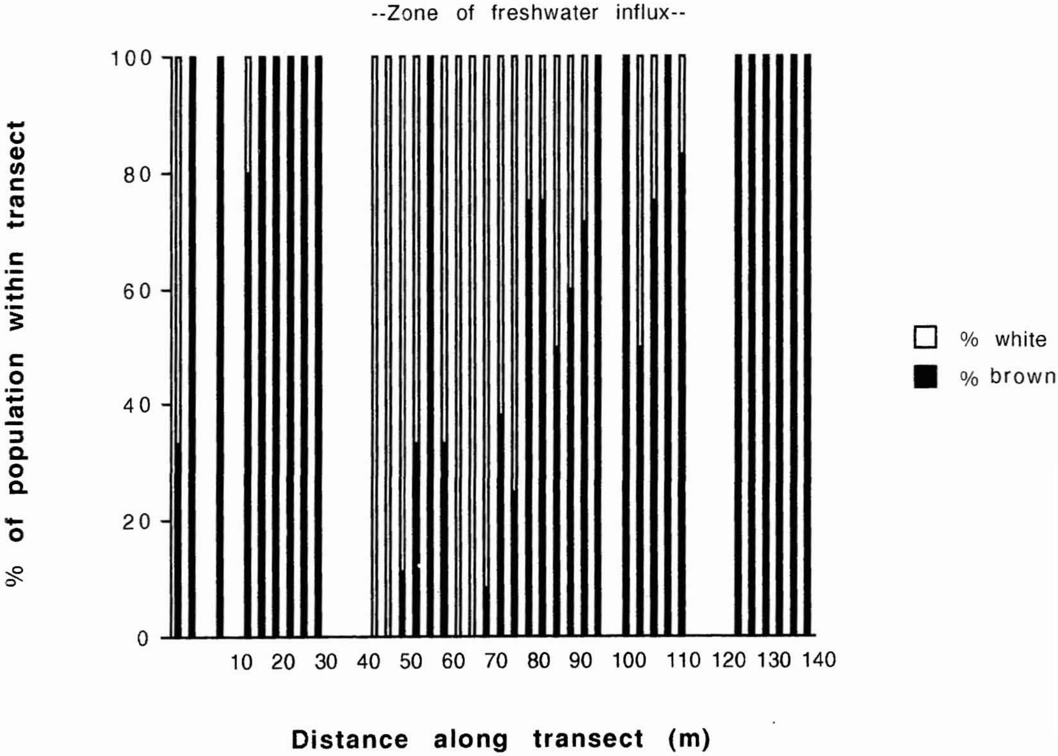

FIGURE 2. Percentages of white and brown *A. elegantissima* m^{-2} along a line transect parallel to shore at a tidal height of -0.15 m at Leo Carrillo State Beach. The area of freshwater flooding is indicated.

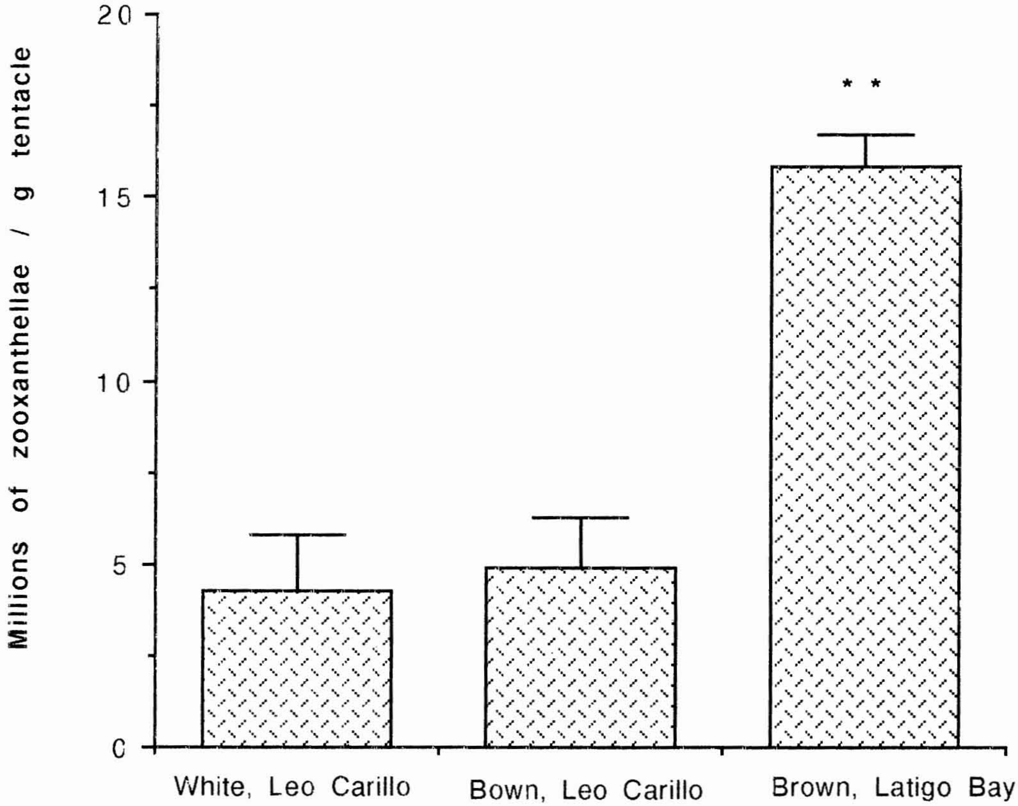

FIGURE 3. Number (with standard errors) of zooxanthellae g^{-1} tentacle in white and brown *A. elegantissima* collected from two sites. Asterisks indicate a significant difference from the white Leo Carillo anemones (student's *t* test).

brown and had significantly greater numbers of zooxanthellae, $15.8 \pm 4.64 \times 10^6$ g^{-1} wet weight of tentacle ($P \ll 0.001$; *t* test).

Exposure to Decreased Salinity

Anthopleura elegantissima placed in 24‰ salinity water (25 : 75% FW : SW) lost 23% of the zooxanthellae in 4 days, whereas *A. elegantissima* in 16‰ (50 : 50% FW : SW) lost 46%, and those in 8‰ (75 : 25% FW : SW) lost 86% of the zooxanthellae after the same time period (Figure 4). *Anthopleura elegantissima* in 24‰ salinity remained open with tentacles fully expanded throughout the experiment. As salinity dropped below 19‰ (60% of normal), anemones in both 16 and 8‰ closed and remained tightly closed, for as long as 9 days, until returned to full-strength seawater at the termination of the treatment.

Based on these data, an equation was calculated predicting the duration of exposure to different salinities until 50% of the zooxanthellae are expelled (Figure 5), showing a direct correlation between decreasing salinity and more rapid loss of zooxanthellae ($R^2 = 1.00$).

Color changes occurred in *A. elegantissima* during hyposaline treatments in the laboratory. Over time, the anemones gradually changed from brown to light brown and finally to dirty white. Such color changes did not occur in the control anemones. Both groups were held under the same illumination, as described in *Materials and Methods*.

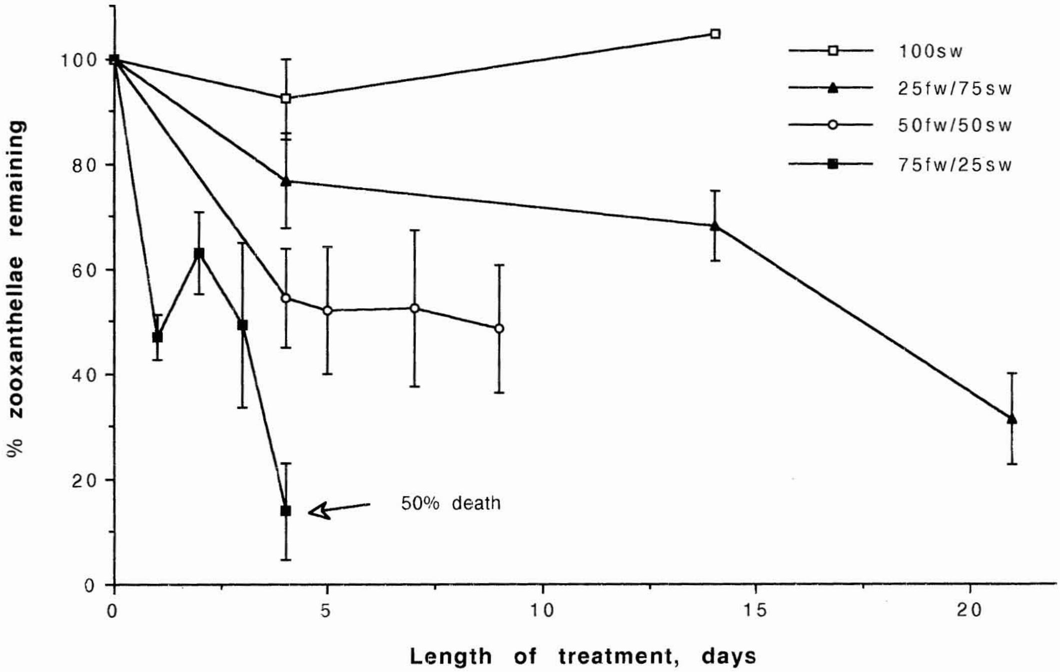

FIGURE 4. Zooxanthellae (with standard errors) lost from *A. elegantissima* tentacles as a function of time immersed in 32, 24, 16, or 8‰ salinity (100, 75, 50, or 25% seawater mixed with fresh water).

Coelenteron Salinity

The salinity within the coelenteron of *A. elegantissima* dropped instantly with external salinity in both the abrupt and gradual salinity reduction experiments (Figures 6, 7). When salinity was abruptly decreased, coelenteron salinity dropped to within 1.34‰ of external salinity in just 45 sec (Figure 6), and when the salinity of the environment was gradually decreased coelenteron salinity was never more than 2.34‰ above the external salinity (Figure 7). Salinity in the coelenteron of *A. elegantissima* sampled at longer intervals was not different from coelenteron salinity of *A. elegantissima* sampled repeatedly at short intervals.

Host Cell Condition: Epifluorescence Microscopy

The fluorescence of host cells surrounding expelled zooxanthellae may provide evidence of a possible mechanism for expulsion (Gates et al. 1992), indicating whether zooxanthellae are released while still within viable host *A. elegantissima* cells. Living host cells show a thin ring of yellow-green fluorescing cytoplasm surrounding the red autofluorescence of chlorophyll in the zooxanthellae (Gates et al. 1992). The control excised tentacle cells appeared yellow-green containing red zooxanthellae, indicating the presence of living host cells. However, the clumps gathered from anemones treated for 4 days with 16‰ salinity water contained no yellow-green flourescence, only isolated zooxanthellae autofluorescing red, in the absence of any living host cell cytoplasm.

DISCUSSION

The color of *Anthopleura elegantissima* is normally greenish brown, in part due to symbiotic algae and in part due to endog-

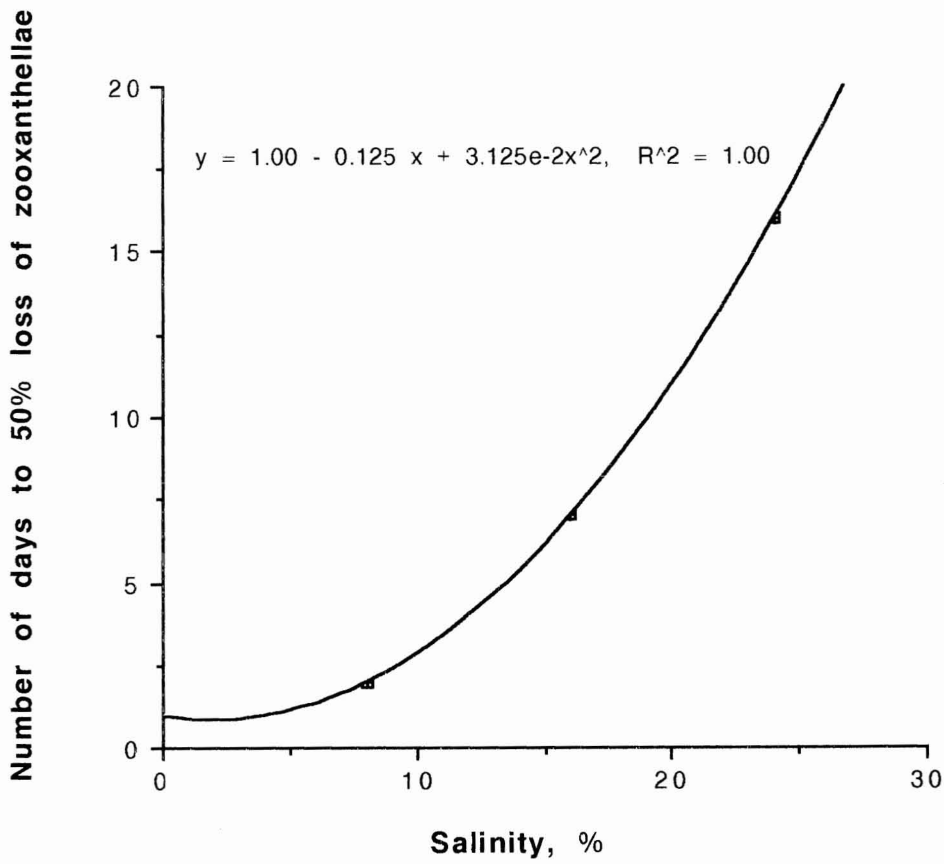

FIGURE 5. Time to loss of 50% of zooxanthellae by *A. elegantissima* as a function of intensity of hyposalinity, indicating an exponential relationship.

enous animal pigments (Buchsbaum 1968). In March of 1992, many *A. elegantissima* in the intertidal zone at LCSB appeared pale. We hypothesized that *A. elegantissima*, a temperate-zone cnidarian, may have responded to an environmental stress by expelling zooxanthellae. The phenomenon of bleaching, or loss of symbiotic zooxanthellae, is of global concern in coral reefs because it indicates a high degree of stress in the ecosystem (Jokiel and Coles 1990). At LCSB, we hypothesized that the environmental stress that led to bleaching was freshwater influx resulting in hyposalinity in the intertidal zone during low tides that followed heavy rains.

Our field observations support this hypothesis. We compared populations of *A. elegantissima* from LCSB with those in a matched control site (LB) that did not experience flooding. Abnormally pale or white *A. elegantissima* were common at LCSB, but were nearly absent at LB (Figure 1), and the greatest density of white *A. elegantissima* occurred within the freshwater inflow area that was the site of flooding at LCSB (Figure 2), whereas there was no correlation between the salinity in transects taken during summer low tides, a nonflood period, and the distribution of white anemones at LCSB.

Tentacles of *A. elegantissima* collected from LCSB, whether white or pale brown, contained dramatically reduced numbers of zooxanthellae compared with controls (Figure 3). The number of zooxanthellae present

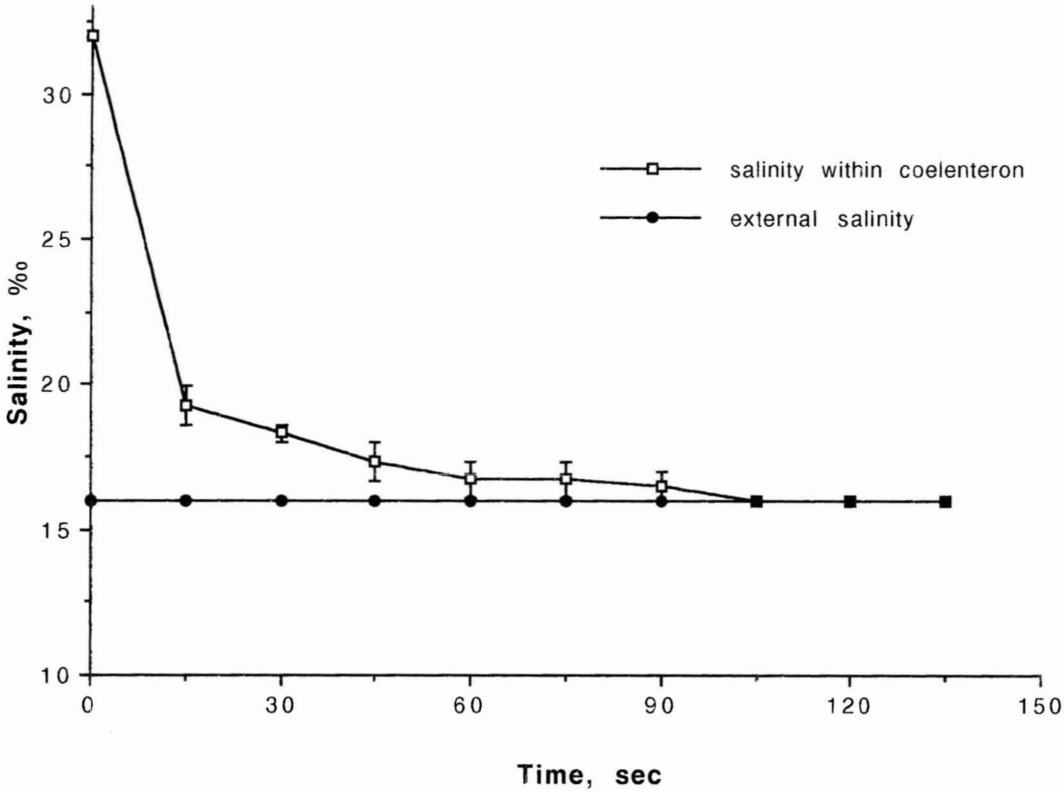

FIGURE 6. Decrease (with standard errors) in coelenteron salinity over time for *A. elegantissima* abruptly placed in water at 16‰ (50% seawater).

in the tissues may indicate the health of the anemone-zooxanthellae symbiosis. Our data indicate that anemones that are paler than normal, even if not fully bleached, may have experienced a dramatic loss of zooxanthellae.

Our laboratory investigations support the hypothesis that hyposalinity causes expulsion of zooxanthellae. The proportion of zooxanthellae lost from *A. elegantissima* in the laboratory was related to the strength and duration of hyposaline exposure (Figure 4). Therefore, in natural flooding situations, a substantial decrease in salinity for repeated short periods of time could have an effect similar to a minimal decrease in salinity for a longer period of time. Even brief periods of hyposalinity, such as flooding during a low tide, could be influential, because decreased salinity dramatically increases the rate of loss of zooxanthellae (Figure 5). At our field site at LCSB, the severity of the intertidal freshwater flooding was compounded by minus low tides that occurred in the days following the heaviest rains, exacerbating the hyposaline influence.

Individual *A. elegantissima* closed tightly when exposed to salinities of 19‰ or less. This reaction has been observed previously in *A. elegantissima* when exposed to other stresses (Pearse 1974, Shick and Dykens 1984), and it is considered a general response to any strong, unfavorable stimulus. The ability of *A. elegantissima* to maintain the salinity within its coelenteron is minimal or absent in the presence of hyposalinity (Figures 6, 7). The lack of ability of *A. elegantissima* to regulate its coelenteron salinity, even for a brief period of time, suggests that environmental hyposalinity is a very potent stress. Because the sessile *A. elegantissima*

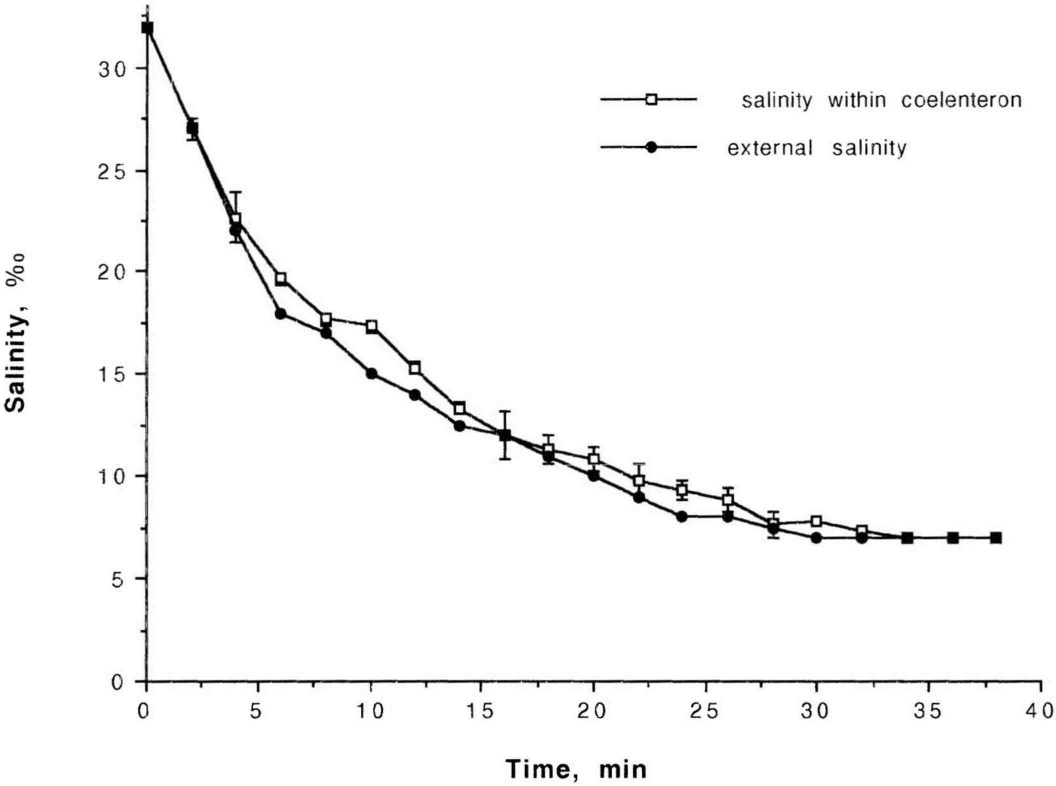

FIGURE 7. Decrease (with standard errors) in coelenteron salinity over time for *A. elegantissima* placed in 32‰ water gradually reduced to 16‰ (50% seawater).

cannot easily move out of the affected area, it has little defense against this situation.

The zooxanthellae expelled by *A. elegantissima* exposed to decreased salinity were not contained in living host cells. We suggest that the mechanism for release of zooxanthellae by the host is necrosis of the host cell caused by hyposaline shock, as speculated by Gates et al. (1992). In contrast, after temperature shock, the sea anemone *Aiptasia* releases the zooxanthellae still contained in whole endoderm cells (Gates et al. 1992). This suggests that, in addition to the loss of the zooxanthellae, *A. elegantissima* may suffer from endodermal tissue damage during hyposaline exposure.

In summary, we have described field observations and laboratory experiments showing that decreased salinity in the environment, even for brief periods, can bring about expulsion of zooxanthellae by temperate-water *A. elegantissima*. Anemones at LCSB were paler in color and contained significantly fewer zooxanthellae per gram of tentacle than *A. elegantissima* in a control site, but had not completely lost their symbionts. We suggest that the expulsion occurs as a result of osmotic water influx into the host cells, causing necrosis. Corals bleached by hyposalinity are able to regain normal numbers of zooxanthellae (Goreau 1964, Egana and DiSalvo 1982), but it is unknown whether *A. elegantissima* is able to recover lost zooxanthellae. Intertidal areas that are subject to flooding with fresh water may be difficult habitats for this species, particularly if the bleached anemones are then more vulnerable to other environmental stresses such as cold shock or bright sunlight. Additional investigations are indicated to understand the way reductions in numbers of zooxanthellae affect *A. elegantissima* and to examine the influence of freshwater influx on anemone population dynamics in nature.

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